

Analysis of Human Cytomegalovirus US3 Gene Products

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Similar to other herpesviruses, human cytomegalovirus remains in the infected host following resolution of the primary infection. The ability to persist in the host after primary infection is believed to be strongly influenced by the ability of HCMV to down-regulate immune recognition of infected cells. One of the genes contributing to immune evasion is the US3 gene. The US3 gene has been shown to retain major histocompatibility complex type I molecules in the endoplasmic reticulum. The US3 gene gives rise to three alternatively spliced RNAs which encode distinct but related proteins. Each of the alternatively spliced transcripts is present early in viral infection, suggesting that the encoded proteins play a role in the viral life cycle. We demonstrate that only the protein encoded by the unspliced US3 transcript is able to retain MHC class I heavy chains in the endoplasmic reticulum. The protein encoded by the singly spliced US3 transcript appears to be processed through the secretory pathway while the protein encoded by the doubly spliced transcript becomes localized to the Golgi apparatus. These experiments raise interesting questions about the functions of the smaller US3 proteins during viral infection in the host. © 2002 Elsevier Science (USA)

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INTRODUCTION

Human cytomegalovirus (HCMV) is a common subclinical viral infection. In immunocompromised individuals and neonates, HCMV infection can result in clinically significant disease and even death (Britt and Alford, 1996). Primary infection is followed by the establishment of a latent or persistent viral infection. Establishment of a latent infection is thought to be facilitated by viral evasion of the immune system.

HCMV has evolved numerous strategies to avoid detection and elimination by the immune system, with different strategies utilized at different time periods during viral replication. Immediately following infection, the virion protein pp65 (ppUL83) interferes with antigenic presentation of the major immediate early proteins (pUL122–123) by an as yet unidentified mechanism (Gilbert *et al.*, 1996). Expression of the immediate early US3 gene results in retention of major histocompatibility complex (MHC) class I protein complexes in the endoplasmic reticulum (ER) (Ahn *et al.*, 1996; Jones *et al.*, 1996). During early times of viral infection, US2 and US11 gene products cause the export of MHC class I complexes into the cytosol where the class I heavy chains are degraded

(Jones *et al.*, 1995; Wiertz *et al.*, 1996a,b). US2 also causes degradation of proteins involved in MHC class II antigen presentation (Tomazin *et al.*, 1999). Another gene product, the US6 gene product, interferes with the transporter for antigen presentation (TAP) activity and loading of peptide antigens onto the MHC class I complex (Hengel and Koszinowski, 1997; Ahn *et al.*, 1997; Sadasivan *et al.*, 1996). Late in viral infection, expression of HLA-E is enhanced by the viral glycoprotein gpUL40, allowing infected cells to avoid destruction by natural killer cells (Tomasec *et al.*, 2000). Additionally, the HCMV gene UL111a encodes a homolog of IL-10, a cytokine with immunosuppressive and anti-inflammatory effects (Kotenko *et al.*, 2000).

The US3 gene is the first viral gene to be transcribed following infection that contributes to immune evasion. Three alternatively spliced mRNA species are generated from the US3 gene; these RNAs encode distinct but related proteins of 3.5, 17, and 21.6 kDa when translated *in vitro* (Weston, 1988; Tenney *et al.*, 1993). All of the predicted protein products have a potential signal peptide; the 21.6-kDa protein also contains a predicted transmembrane domain. The 17- and 21.6-kDa proteins have 134 amino acids in common, including two cysteine residues and a glycosylation signal present in exon 3. The 17- and 21.6-kDa proteins are modified by glycosylation when translated *in vitro*, in the presence of canine pancreatic microsomes. Expression of the US3 gene in different cell types has demonstrated that US3 expression delays the maturation of MHC class I molecules by

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retaining these proteins in the ER (Jones *et al.*, 1996; Ahn *et al.*, 1996). Coprecipitation of a 22-kDa US3 protein with MHC class I molecules suggests that this protein is critical for retention of MHC class I heavy chains in the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996). Work by Lee *et al.* (2000) further suggests that the ER luminal and transmembrane domains of the 22-kDa US3 protein are sufficient for retention of MHC class I molecules. The possible contributions of the smaller US3 proteins to MHC class I molecule retention were not analyzed in these studies (Jones *et al.*, 1996; Ahn *et al.*, 1996; Jun *et al.*, 2000). In this article we examined the effects of the individual US3 proteins on MHC class I molecules. Analysis of cell lines expressing individual US3 proteins demonstrated that the large US3 protein alone is sufficient for MHC class I molecule retention. We further demonstrate that the singly spliced US3 transcript encodes a protein of 17 kDa; this protein appears to traffic through the secretory pathway. Several interesting questions still remain to be answered, including the roles of the 17- and 3.5-kDa proteins in viral infection.

RESULTS

Expression of the US3 gene

The US3 gene has been defined as an immediate early gene, with transcription of the gene occurring following infection in the absence of *de novo* protein synthesis (Weston, 1988). Similar to many of the other immediate early genes of HCMV, the US3 gene encodes multiple alternatively spliced transcripts: a full-length unspliced transcript, a singly spliced transcript, and a doubly spliced transcript (Fig. 1A) (Tenney *et al.*, 1993). The US3 transcripts appear by 1 h postinfection, with peak accumulation of transcripts occurring at 3 hpi, followed by a marked decline in transcript levels by 5 hpi (Biegelke, 1995; Greijer *et al.*, 2001). Isolation of cDNAs clones representative of each of the transcripts suggests that the spliced RNAs accumulate preferentially in the absence of protein synthesis and further suggests that the full-length transcript is the most abundant US3 message (Tenney *et al.*, 1993). We used RNase protection assays to determine the relative abundance of each of the alternatively spliced US3 transcripts at various times following HCMV (Towne) infection of human diploid fibroblasts. An antisense radiolabeled probe (see Fig. 1A) generated from the HCMV strain AD169 full-length cDNA was used for the detection of the US3 transcripts. The protected RNA fragments were predicted to be 291, 251, or 161 nucleotides in length, corresponding to the unspliced, singly spliced, or doubly spliced transcripts, respectively. An RNA probe protected by glyceraldehyde phosphate dehydrogenase transcripts was used as an internal standard.

US3 transcripts were first detected 1 h postinfection with the level of transcripts increasing until 3 to 4 hpi

(Fig. 1B). By 5 hpi, all three forms of the US3 RNAs were beginning to decline in abundance, confirming the Northern blot analysis of US3 transcript levels (Biegelke, 1995). Quantitative analysis, comparing the amounts of the protected US3 fragments to levels of the cellular message, GPDH, demonstrated that the unspliced US3 transcript was the most abundant message at 1–3 h of infection (Fig. 1C). The levels of the singly spliced transcript were somewhat lower than those of the full-length transcript; the levels of these two transcripts varied with similar kinetics (Fig. 1C). In contrast to the full-length and singly spliced transcripts, the doubly spliced transcript accumulated and declined with delayed kinetics compared to the larger transcripts. The level of the doubly spliced transcripts was maximal at 4 hpi, declining slightly in abundance by 5 hpi (Figs. 1B and 1C). All three alternatively spliced forms of the US3 transcripts were clearly present early in infection, suggesting that the encoded proteins play a role in viral infection in the infected host.

In the RNase protection assays, the sizes of the protected fragments corresponding to the full-length and singly spliced transcripts were smaller than predicted by approximately 15 nucleotides. Comparison of RNase protection assay results obtained using RNA from cells that contain HCMV strain Towne or strain AD169 US3 sequences revealed differences in the sizes of the protected fragments (see Fig. 2). To determine the basis of the size variation in the protected fragments, the HCMV strain Towne US3 gene was sequenced. Seven nucleotide changes were identified, with three of the nucleotide changes located near the 3' end of the probe used for the RNase protection assays (Fig. 1A). The three-nucleotide mismatch resulted in additional digestion of the RNA probe when hybridized to Towne US3 transcripts, resulting in a decrease of 15 bp in the protected fragment, accounting for the discrepancy between the predicted and observed sizes of the protected fragments. Five of the nucleotide changes seen in HCMV strain Towne resulted in amino substitutions in the encoded US3 proteins (see Table 1 and Fig. 1A). None of the amino acid changes were in the signal peptide or transmembrane domain.

The singly spliced US3 transcript encodes a 17-kDa protein

Two polypeptides of 22 and 17 kDa were detected in US3-expressing cell lines and virally infected cells (Ahn *et al.*, 1996; Jones *et al.*, 1996). The 17-kDa peptide was postulated to be either the translation product of a spliced US3 transcript, or alternatively, the immature form of the 22-kDa protein. To definitively identify the protein products encoded by the unspliced and singly spliced US3 RNAs, a number of cell lines were generated that express US3 sequences. To generate the cell lines, US3 cDNAs (from strain AD169), a truncated cDNA

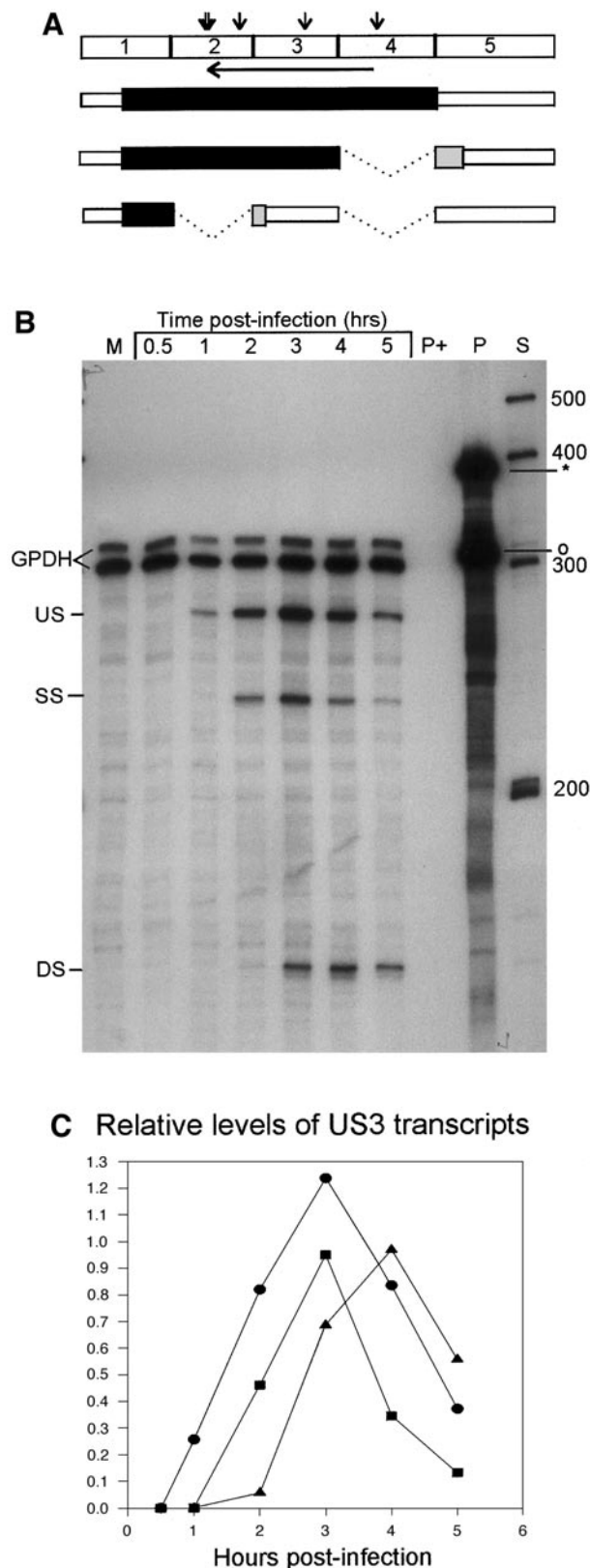


FIG. 1. Analysis of US3 expression. (A) Diagram of the US3 transcription unit. Numbered rectangles represent exons; vertical arrows depict positions of amino acid changes; the horizontal arrow corresponds to the probe used for RNase protection assays; open rectangles represent 5' and 3' untranslated regions of the US3 transcripts; gray rectangles represent protein coding regions; shared amino acids are

corresponding to the unspliced transcript but lacking exon 5, or the genomic US3 sequence (from strain Towne and equivalent to the cDNA corresponding to the unspliced transcript), were inserted into the plasmid vector pIRES-neo. Plasmids were transfected into the U373 glioblastoma cell line and neomycin-resistant cell clones were isolated. The resulting cell lines were analyzed for US3 transcripts using RNase protection assays; a minimum of four different cell lines constructed with each of the US3-expression plasmids was analyzed for US3 transcripts.

RNase protection analyses of RNA isolated from the cell lines containing the cDNA corresponding to the AD169 unspliced transcript (US-cDNA) or the Towne genomic US3 sequences revealed two protected fragments corresponding to the unspliced and singly spliced US3 transcripts (Fig. 2A, Lanes 9–12). The strain-specific variation in the size of the protected fragments resulted from nucleotide changes as discussed above. Somewhat surprisingly, the doubly spliced transcript was not detected in cell clones expressing either the AD169 US-cDNA or the Towne genomic sequences. Analyses of cells containing the AD169 US3 US-cDNA also revealed cell clone-specific protected fragments that are presumed to correspond to aberrantly spliced RNAs (Fig. 2A).

A predominant fragment corresponding to the unspliced US3 transcript was protected using RNA from cell lines expressing the truncated cDNA composed of exons 1 through 4 (US3 Δ ex5; Fig. 2A, Lanes 7 and 8). Spliced US3 transcripts in these cell lines were predicted to be degraded through the nonsense-mediated degradation pathway. Detection of only a low level of the protected fragments corresponding to the singly spliced transcript confirmed this prediction. RNase protection assays of RNA from cell clones containing the cDNA generated from the singly spliced (SS-cDNA) or the dou-

indicted by the black rectangles; dotted lines indicate splicing events (Tenney *et al.*, 1993; Weston, 1988). (B) RNase protection assays were performed on whole-cell RNA isolated from mock-infected (M) or from HCMV strain Towne-infected human diploid fibroblasts at the indicated times postinfection (h). The probes (P) for the internal standard, glyceraldehyde phosphate dehydrogenase (GPDH), and US3 transcripts are indicated by an * and °, respectively. P+, yeast tRNA and the probes treated with the RNase mixture; GPDH, protected fragments corresponding to levels of the cellular message; US, SS, and DS, protected fragments corresponding to unspliced, singly spliced, or doubly spliced US3 transcripts, respectively. S, RNA molecular weight markers with the size indicated in nucleotides. (C) Quantification of US3 transcripts. Protected fragments corresponding to US, SS, and DS US3 transcripts were quantified by a scanning densitometer using Molecular Dynamic software. Levels of US3 transcripts were determined by normalizing to the density of the smaller GPDH protected fragment and correcting for the number of U residues present in the protected fragment. Levels of US3 transcripts were plotted vs hours postinfection. ●, unspliced US3 mRNA; ■, singly-spliced US3 mRNA; and ▲, doubly-spliced US3 mRNA.

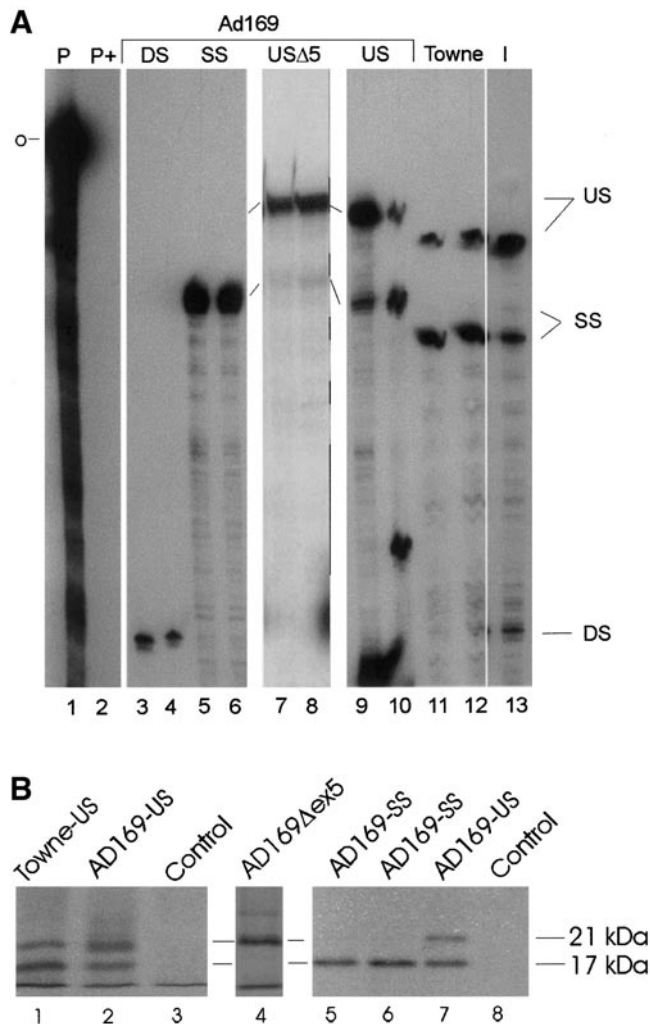


FIG. 2. (A) RNase protection analyses of representative cell lines expressing US3 transcripts. Stable US3-expressing U373 cell lines were established using the pIRESneo-derived plasmids, pBJ317 [which expresses the cDNA corresponding to the unspliced AD169 US3 transcript (US)], pBJ380 [which expresses a truncated cDNA derived from the unspliced AD169 US3 transcript with exon 5 deleted (USΔ5)], pBJ318 [which expresses the cDNA corresponding to the singly spliced AD169 US3 transcript (SS)], pBJ319 [which expresses the cDNA corresponding to the doubly spliced US3 transcript (DS)], or pB320 [which expresses the Towne US3 genomic sequence (Towne)]. The probe depicted in Fig. 1 was used to detect US3 transcripts in total RNA isolated from the stable cell lines. Probe fragments corresponding to the unspliced (US), singly spliced (SS), and doubly spliced transcripts (DS) are indicated. Lane 1, probe alone (P); Lane 2, probe plus RNases (P+); Lane 3, RNA from cell line 319-1; Lane 4, RNA from cell line 319-2; Lane 5, RNA from cell line 318-4; Lane 6, RNA from cell line 318-6; Lane 7, RNA from cell line 380-3; Lane 8, RNA from cell line 380-4; Lane 9, RNA from cell line 317-B2; Lane 10, RNA from cell line 317-1; Lane 11, RNA from cell line 320-4; Lane 12, RNA from cell line 320-1; Lane 13, RNA from HCMV strain Towne-infected HDF's 4 h postinfection (I). (B) Analysis of US3 proteins in the U373 cell lines. The stable US3-expressing cell lines were radiolabeled using [³⁵S]methionine-cysteine (EasyTag, New England Nuclear) prior to immunoprecipitation with polyclonal antiserum to US3 proteins (kindly provided by T. Jones). A minimum of four individual cell lines derived by introduction of the indicated plasmid into U373 cells was analyzed; representative data are presented for the indicated cell lines. Lane 1, cells expressing the

TABLE 1

US3 Amino Acid Variation		
Amino acid position	AD169	Towne
52	Y ^a	H
53	F	Y
68	N	S
106	T	P
150	M	I

^a Single letter amino acid code.

bly spliced transcript (DS-cDNA) detected protected fragments of the predicted sizes, 251 and 161 nucleotides, respectively, that correspond to singly and doubly spliced RNAs (Fig. 2A, Lanes 3–6). Control cell lines that contained the plasmid vector but no US3 sequences did not yield any protected fragments corresponding to US3 transcripts (data not shown).

US3 proteins present in the cell lines were analyzed by immunoprecipitation experiments, using polyclonal antisera to amino acids 33 to 113 of the US3 open reading frame (Jones *et al.*, 1996). Cells expressing the entire US3 open reading frame (either the AD169 US-cDNA or the Towne genomic sequence) contained two US3 proteins of 22 and 17 kDa (Fig. 2B, Lanes 1, 2, and 7). Cell lines expressing the SS-cDNA synthesized a US3 protein of 17 kDa (Fig. 2B, Lanes 5 and 6). Cell lines containing the truncated cDNA (lacking exon 5) synthesized a single US3 protein of 22 kDa (Fig. 2B, Lane 4). These data demonstrated that the 22-kDa protein is encoded by the unspliced US3 RNA and that the 17-kDa US3 protein is the translation product of the singly spliced transcript. Although we constructed cell lines that express the doubly spliced US3 transcript (Fig. 2A, Lanes 3 and 4), we were unable to demonstrate synthesis of the encoded protein as no reagents are currently available that react with the predicted peptide. By comparison with the cell clones expressing other forms of the US3 RNAs, we expect that the 3.5-kDa protein is synthesized in these cells. No proteins were precipitated from the control cell lines with the anti-US3 antiserum (Fig. 2B, Lanes 3 and 8).

Functional analysis of the US3 proteins

The involvement of the different US3 proteins in retention of MHC class I molecules in the ER was assayed using the cell lines described above. To determine the

Towne US3 gene (cell line 320-4); Lane 2, cells expressing US3 sequences corresponding to the AD169-unspliced transcript (cell line 317-B2); Lane 3, control cells with no US3 sequences (cell line GFP-4); Lane 4, cells expressing US3 sequences corresponding to AD169 exons 1–4 (AD169Δex5, cell line 380-3); Lanes 5 and 6, cells expressing the cDNA corresponding to the singly spliced AD169 transcript (Lane 5, cell line 318-5; Lane 6, cell line 318-6); Lane 7, cell line 317-B2; Lane 8, cell line GFP-4.

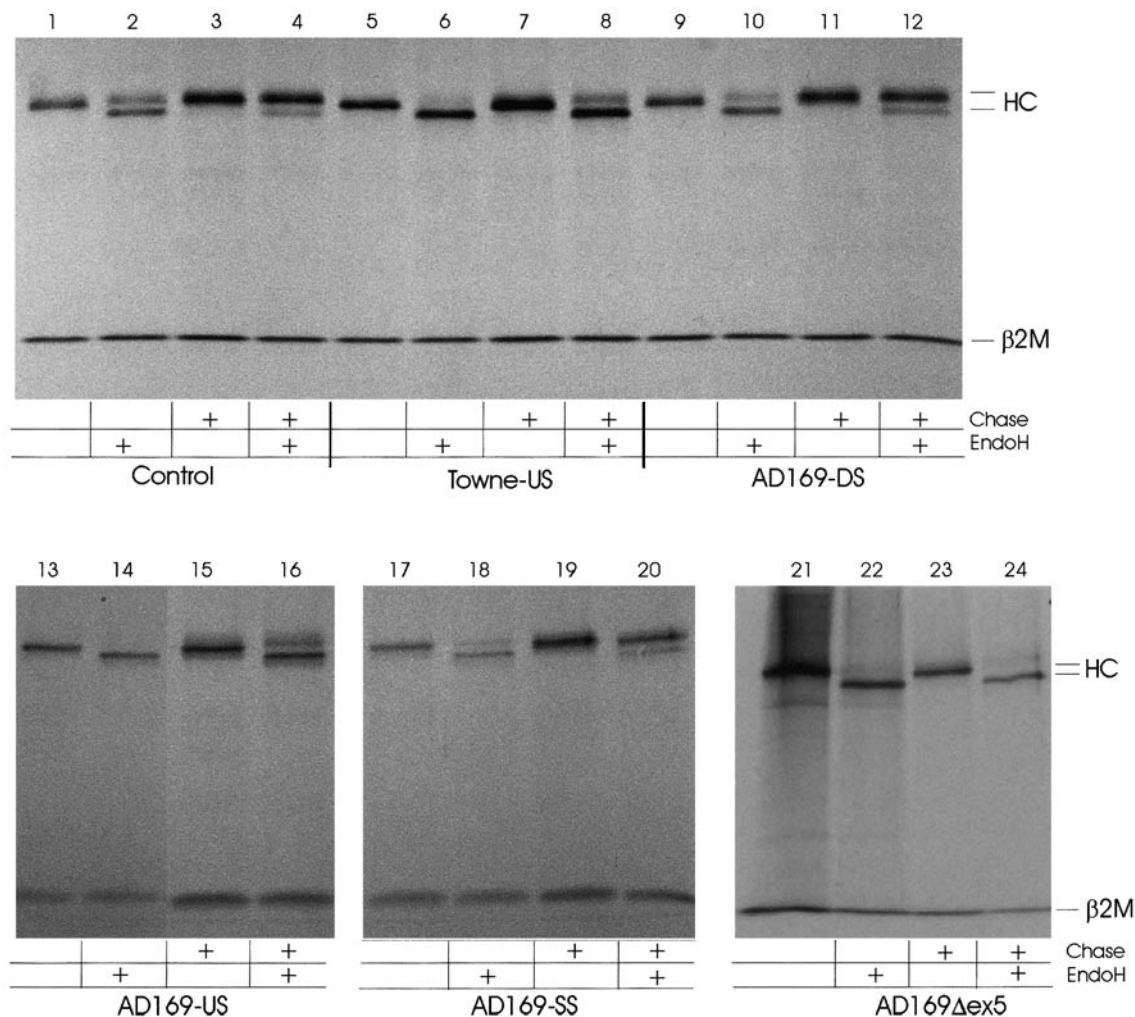


FIG. 3. Retention of MHC class I molecules by US3 proteins. Cells constitutively expressing the alternatively spliced transcripts of the US3 gene were radiolabeled with [35 S]methionine, and then either immunoprecipitated with a monoclonal antibody to MHC class I molecules (W6/32) or subjected to a 2-h chase with nonradioactive amino acids (Chase) prior to immunoprecipitation. Following immunoprecipitation, complexes were divided into two aliquots and either mock-digested or digested with endoglycosidase H (EndoH). Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. AD169-US, cell line 317-B2, which expresses the cDNA corresponding to the AD169-unsliced US3 transcript, Lanes 13–16; AD169-US Δ ex5, cell line 380-3 which expresses the AD169 unsliced cDNA with exon 5 deleted, Lanes 21–23; cell line 318-6 expresses the cDNA corresponding to the singly spliced AD169 US3 transcript, Lanes 17–20; Control, cell line GFP-4, which expresses GFP, Lanes 1–4; Towne-US, cell line 320-4, which expresses the HCMV strain Towne genomic sequence, Lanes 5–8; and AD169-DS, cell line 319-4 which expresses the cDNA corresponding to the doubly spliced AD169 US3 transcript, Lanes 9–12. Four individual cell lines derived from each of the plasmids; data from representative cell lines are shown above. HC, MHC class I heavy chains; β 2M, beta-2-microglobulin.

effects of the US3 proteins on the maturation of MHC class I molecules, cells were treated with a pulse of radioactive amino acids and then either harvested immediately or subjected to a chase with nonradioactive amino acids prior to harvest. Cell lysates were immunoprecipitated with monoclonal antibody W6/32, an antibody that recognizes the MHC class I complex. The immunoprecipitates were divided into two aliquots and either treated with endoglycosidase H or mock-treated. Oligosaccharide chains present on proteins retained in the ER are sensitive to endoglycosidase H digestion; removal of the oligosaccharides by endoglycosidase H is indicated by a decrease in protein size. Four different cell

lines constructed with each of the US3-expression plasmids were analyzed for MHC class I molecule retention; representative data from one of each of the cell lines analyzed is shown in Fig. 3.

In all of the cell lines examined, after a 30-min labeling period, the majority of the MHC class I molecules was sensitive to endoglycosidase H treatment. This reflected the *de novo* synthesis of the proteins and insufficient time for the proteins to traffic from the ER to the Golgi. In the control cell line, a 2-h chase with nonradioactive amino acids was sufficient for processing of the MHC class I molecules and associated maturation of the oligosaccharide chains, making them largely resistant to

endoglycosidase H digestion (Fig. 3, compare Lanes 1–4). In contrast, in cells expressing the 17- and 22-kDa US3 proteins, the MHC class I heavy chains remained predominantly endoglycosidase H sensitive following the chase period (Fig. 3, Lanes 5–8 and 13–16). Similarly, in the cell line expressing only the 22-kDa US3 protein, the oligosaccharides on the MHC class I heavy chains remained largely sensitive to endoglycosidase H digestion, indicating retention of the proteins in the ER (Fig. 3, compare Lanes 21–24). These data demonstrated that expression of the 22-kDa US3 protein alone was sufficient for retention of MHC class I molecules in the ER.

The 17-kDa US3 protein and the 22-kDa have a large number of amino acids in common, suggesting that the 17-kDa protein, similar to the 22-kDa protein, could potentially have an effect on maturation of MHC class I molecules. Pulse-chase experiments were also performed with the cell lines expressing only the 17-kDa protein. Following the chase period, the MHC class I heavy chains became endoglycosidase H resistant, similar to the control cells (Fig. 3, Lanes 17–20), demonstrating that the 17-kDa protein is unable to retain MHC class I molecules in the ER. Coprecipitation experiments demonstrated that the 17-kDa protein does not physically associate with MHC class I molecules, confirming the results of Ahn *et al.* (1996) and Jones *et al.* (1996). Likewise, expression of the doubly spliced transcript had no effect on the maturation of MHC class I molecules (Fig. 3, Lanes 9–12). These data demonstrated that the domain for retaining MHC class I heavy chains in the ER is not present in the 134 amino acids that are common between the 17- and 22-kDa proteins. Thus, the domain for retaining MHC class I molecules in the ER resides in the COOH-terminal 52 amino acids of the 22-kDa US3 protein. The COOH-terminal amino acids also appear to be critical for the protein–protein interactions as only the 22-kDa protein coprecipitates with the MHC class I molecules (Jones *et al.*, 1996; Ahn *et al.*, 1996) and the physical association appears to play an important role in alteration of the MHC class I molecule processing (Zhao *et al.*, unpublished data).

Intracellular distribution of US3 proteins

Immunofluorescence analysis of the intracellular location of US3 proteins identified the proteins as residents of the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996). Lee *et al.* (2000) identified the ER luminal portion of the 22-kDa US3 protein as containing the intracellular ER localization signal. This contrasts with other ER resident proteins where retrieval to the ER is a function of the amino acid sequence in the cytoplasmic tail of the protein. This suggested that the 17- and the 22-kDa US3 proteins would both localize to the ER as they have 134 amino-terminal amino acids in common, which includes the majority of the ER luminal domain of the 22-kDa protein

(See Fig. 1A). We examined the intracellular location of the US3 proteins by expressing US3-enhanced green fluorescent protein (EGFP) fusion proteins, in which the carboxyl-terminal end of the US3 protein was extended with the open reading frame of EGFP. The cDNAs representing each of the spliced variants of the US3 transcripts was inserted in-frame with EGFP; the resulting plasmids were transfected into HeLa cells. Indirect immunofluorescence was used to label the ER or the Golgi apparatus, using antibodies to protein disulfide isomerase, a resident luminal ER protein, or to human golgin-97, a resident protein of the Golgi apparatus, respectively. Transfected cells were identified by green fluorescence; nuclei were stained with DAPI and visualized by their blue fluorescence. Brefeldin A treatment was used to identify proteins trafficking through the Golgi and *trans*-Golgi network. Brefeldin A disrupts the Golgi apparatus, resulting in a rapid relocation of proteins in the Golgi to the ER (Sciaky *et al.*, 1997). Fluorescence micrographs of representative fields containing transfected cells are shown in Fig. 4.

The 22-kDa US3-EGFP protein was predominantly localized to the ER as demonstrated by colocalization of US3-EGFP with PDI, and by the lack of a Brefeldin A effect on the pattern of green fluorescence (Figs. 4B and 4C), confirming and extending published reports (Jones *et al.*, 1996; Ahn *et al.*, 1996; Lee *et al.*, 2000). The 17-kDa EGFP fusion protein, although found partially in the ER, was also located in the Golgi and in vesicles in the cytoplasm (Fig. 4). The pattern of green fluorescence was disrupted by Brefeldin A treatment, which resulted in localization of the green fluorescence to the ER. These data suggested that the 17-kDa US3 protein is processed through the secretory pathway. The putative 3.5-kDa US3 protein was also expressed as an EGFP-fusion protein. In contrast to the results observed with the larger US3 proteins, the 3.5-kDa US3-EGFP fusion protein localized to the Golgi apparatus (Fig. 4). Brefeldin A treatment disrupted the pattern of 3.5-kDa US3-EGFP fluorescence, redistributing the green fluorescence to a pattern that colocalized with PDI (Fig. 4C). Addition of EGFP to the 3.5-kDa protein is a significant modification of the US3 protein; generation of reagents able to specifically detect the 3.5-kDa protein is needed to determine whether the native protein localizes to the Golgi apparatus.

DISCUSSION

Retention of MHC class I heavy chains in the ER is mediated through the HCMV US3 gene, and specifically, the 22-kDa US3 protein. However, the relative abundance of all three transcripts encoded by the US3 gene suggests that each of the encoded proteins contributes to the viral life cycle in the infected host. Comparison of the levels of the different US3 transcripts, particularly in cell lines expressing the entire US3 gene, suggests that

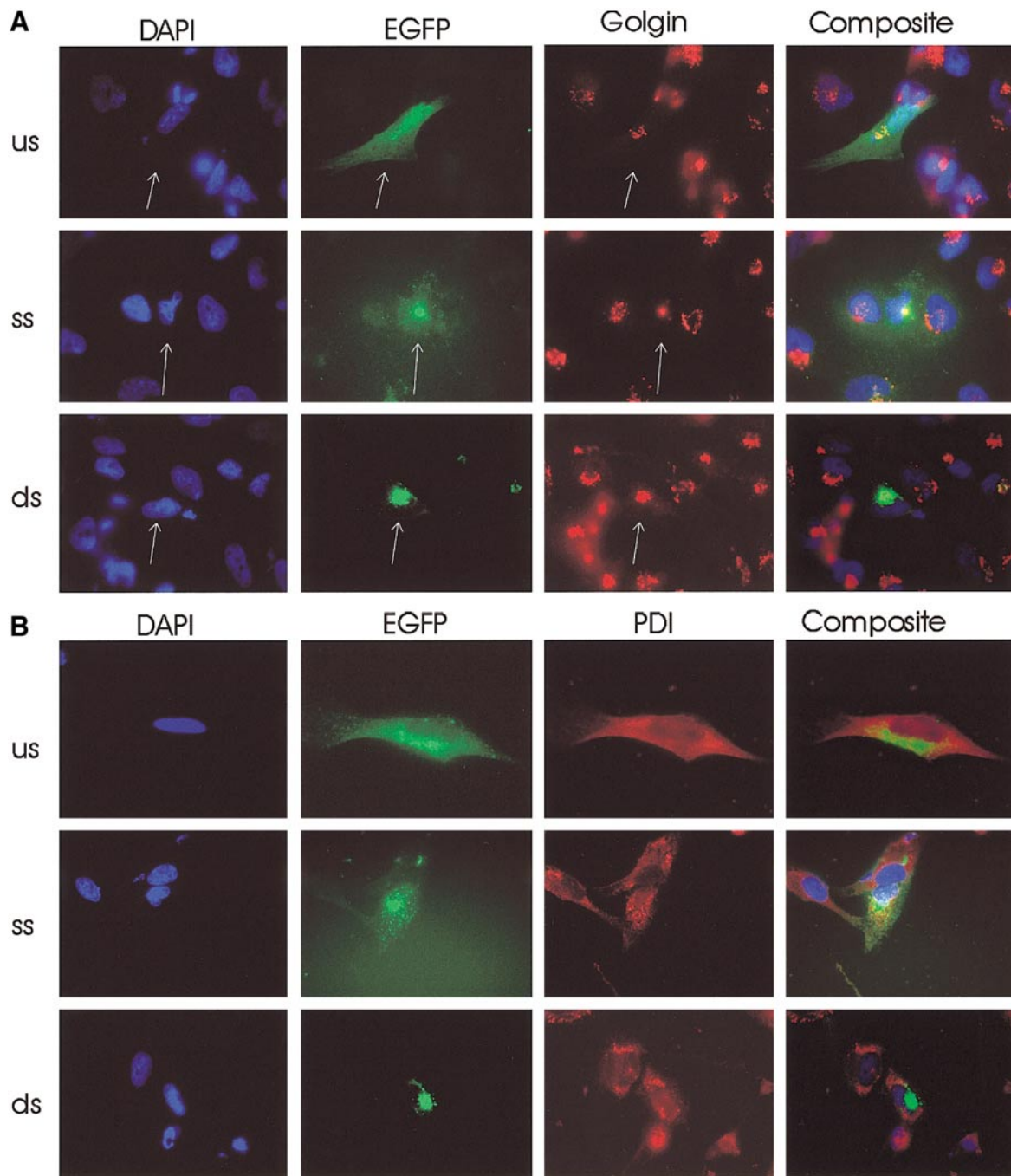


FIG. 4. Fluorescence microscope localization of US3-EGFP fusion proteins. Cells were transfected with plasmids expressing the 22 kDa (us), 17 kDa (ss), or 3.5 kDa (ds) US3 proteins as EGFP-tagged proteins. Cells were either left untreated (A, B) or were treated with Brefeldin A (C). Nuclei were stained with DAPI and visualized using blue fluorescence (DAPI); the resident ER protein, protein disulfide isomerase (PDI; B and C), and the resident Golgi apparatus protein, golgin-97 (A) were detected using indirect immunofluorescence with the secondary antibody conjugated to Texas red (PDI). The composite images are the sum of the blue, green, and red fluorescence. Cells are magnified $\times 1000$.

splicing of the primary US3 transcript is regulated, perhaps by splice site repressors or by viral proteins. The experiments presented above are the first to demonstrate that proteins encoded by alternatively spliced forms of US3 transcripts do not play a role in inhibiting the maturation of the MHC class I heavy chains. The function of the US3 gene and the 22-kDa protein in

immune evasion is strain-independent despite amino acid variations in the open reading frames of HCMV strains AD169 and Towne.

The 17-kDa US3 protein is highly related to the 22-kDa protein; the two proteins differ only at the COOH end, including the absence of a transmembrane domain in the 17-kDa protein. The 17-kDa protein was unable to retain

C Brefeldin A-treated samples

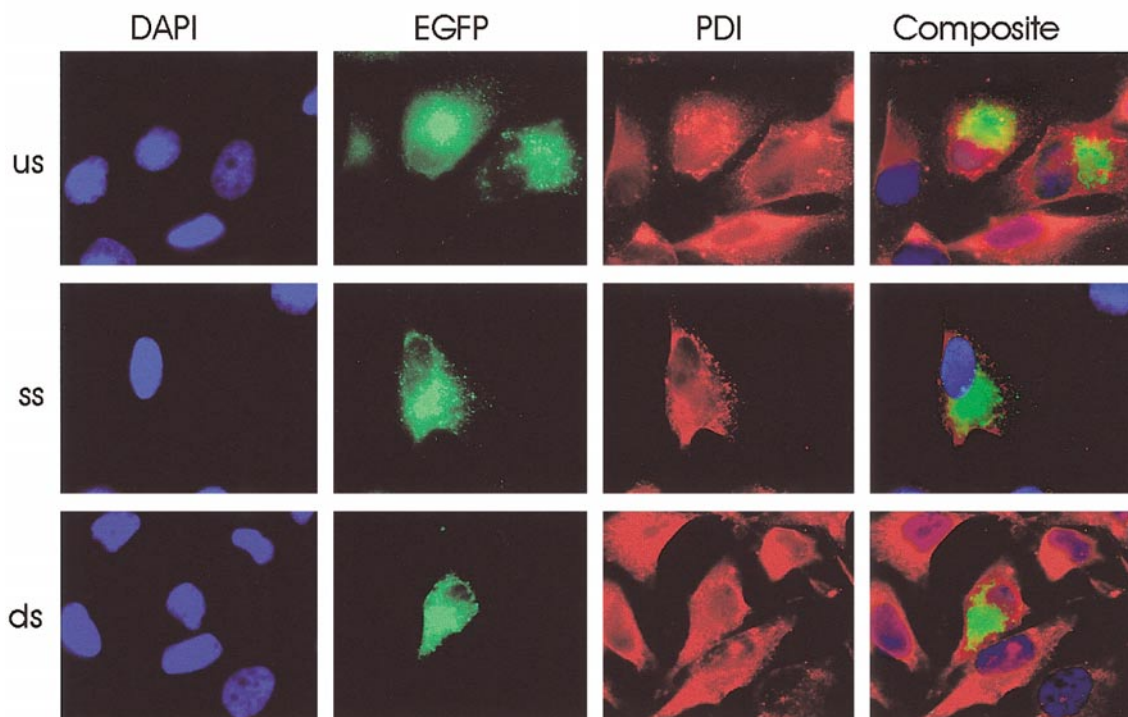


FIG. 4—Continued

MHC class I molecules in the ER and also does not coprecipitate with MHC class I molecules (Jones *et al.*, 1996; Ahn *et al.*, 1996; Zhao *et al.*, unpublished data). These data support the conclusion of Lee *et al.* (2000), where the US3 transmembrane domain is required for retention of MHC class I molecules in the ER. The 17-kDa protein appeared to be processed through the secretory pathway, suggesting that the COOH-terminal 52 amino acids unique to the 22-kDa protein contain an ER retention signal. A function for the 17-kDa protein has yet to be identified.

Evidence for *in vivo* synthesis of the 3.5-kDa US3 protein is still lacking. The RNase protection assays clearly indicate that the transcript encoding this predicted protein is expressed during viral infection. Expression of the open reading frame of the 3.5-kDa protein as an EGFP-tagged protein resulted in localization of the green fluorescence to the Golgi apparatus. The localization of the fusion protein is surprising and intriguing. The 3.5-kDa EGFP fusion protein lacks a transmembrane domain characteristic of enzymes that localize to the Golgi. Identification and localization of the 3.5-kDa protein during infection are needed to confirm the results seen with the EGFP-tagged protein.

Alteration of antigen presentation by MHC class I molecules is a common strategy used by viruses to evade detection by the host immune system. The adenovirus E3-19k ER resident protein retains MHC class I molecules in the ER through the interaction of the E3-19k

luminal and transmembrane domains with MHC class I molecules (Gabathuler *et al.*, 1990). Murine cytomegalovirus (MCMV) encodes proteins that also interfere with the presentation of antigens by MHC class I molecules. The MCMV gene, m152, encodes a glycoprotein of 40 kDa that selectively retains mouse MHC class I heavy chains in the ER-Golgi network (Ziegler *et al.*, 1997). A secreted form of the m152 protein is able to bind to MHC class I molecules (Ziegler *et al.*, 2000), in contrast with the results presented here for US3 proteins. Deletion of m152 attenuates MCMV, underscoring the importance of immune evasion genes in the life cycle of the virus in the natural host.

Given the likely importance of the US3 gene for viral replication in the infected host, the short half-life of the US3 22-kDa protein (<60 min) (Ahn *et al.*, 1996; Jones *et al.*, 1996), and the dramatic transcriptional down-regulation of US3 expression early in infection are surprising. Although US3 expression does not interfere with the maturation of a cellular glycoprotein, the transferrin receptor, it is possible that maturation of viral glycoproteins may be inhibited by continuous expression of the 22-kDa US3 protein. Alternatively, continual expression of the 17- and 3.5-kDa proteins may have deleterious effects on viral replication.

The ability of the HCMV 22-kDa US3 protein to down-regulate expression of the MHC class I heavy chains is predicted to play an important role in the establishment of infection in the host. The recent identification of the

role of MHC class I molecules in development of the nervous system (Huh *et al.*, 2000) suggests that HCMV infection of the fetus with associated US3 expression may interfere with normal brain development, possibly contributing to the mental retardation seen in severely affected infants.

MATERIALS AND METHODS

Cells, virus, and transfections

Human glioblastoma cells (U373 cells) were obtained from Adam Geballe, Fred Hutchinson Cancer Research Center (Seattle, WA); HeLa cells were obtained from Clontech (Palo Alto, CA) and primary human diploid fibroblast cultures were established as previously described (Biegalka, 1995). Human diploid fibroblasts, HeLa, and U373 cells were propagated in Dulbecco's minimal essential medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% NuSerum (Collaborative Research Products, Bedford, MA), penicillin, streptomycin, and glutamine. HCMV strain Towne was propagated in primary human fibroblasts.

Transfections were performed using calcium phosphate. Briefly, cells (80–90% confluent) were fed with the appropriate medium the day of the experiment. Plasmid DNA (10 μ g) in a mixture of 0.125 M CaCl_2 and 1 \times BBS (50 mM BES pH 6.95, 280 mM NaCl, and 5 mM Na_2HPO_4) was added to the medium overlaying the cells. Following an overnight incubation in 3.5% CO_2 at 35°C, the cells were washed and fed with complete medium. U373 cell clones containing stable plasmid integrants were selected using complete medium containing 500 μ g/ml G418.

RNA analysis

Total cellular RNA was isolated from infected cells or stable transfected cell lines using acid-phenol guanidium (Chomczynski and Sacchi, 1987). RNase protection assays were performed using the RPA II kit (Ambion, Austin, TX) as recommended by the manufacturer; the products of the assays were analyzed on 5% denaturing polyacrylamide gels. For the synthesis of radiolabeled RNA complementary to US3 messages, a 285-bp *SphI-KpnI* fragment of the US3 gene was inserted into pBS+ (Stratagene, La Jolla, CA) to generate pBJ335. Radiolabeled antisense US3 RNA was transcribed using the Maxiscript T7 kit (Ambion) and ^{32}P -UTP. Century templates (Ambion) were transcribed to generate RNA molecular weight markers; an antisense glyceraldehyde phosphate dehydrogenase (GPDH) transcript was used as an internal standard. Quantification of the protected US3 RNA fragments was performed using a scanning densitometer and Molecular Dynamics software, normalizing the amounts of the US3 transcripts to the cellular message, GPDH.

Sequence analysis

The DNA sequence of the HCMV strain Towne US3 open reading frame was determined by sequence analysis of pBJ174 (Biegalka, 1999) using gene-specific primers and the Sequenase kit (USB, Cleveland, OH) or the Big Dye terminator kit (Perkin-Elmer, Foster City, CA). The sequence of the HCMV strain Towne US3 open reading frame is present in GenBank as Accession No. AY038933.

Plasmids

pBJ317, pBJ318, pBJ319, and pBJ320 were constructed by inserting the cDNAs corresponding to the HCMV strain AD169 unspliced, singly spliced, or doubly spliced US3 transcripts (isolated from plasmids p516, p517, p518 kindly provided by A. Colberg-Poley) (Tenney *et al.*, 1993) or the Towne US3-coding region (isolated from pBJ174) (Biegalka, 1999), respectively, into the multiple cloning site of pIRESneo (Clontech, Palo Alto, CA). pEGFP-IRESneo was used to generate control cell lines. Plasmid DNA was prepared by alkaline lysis and cesium chloride gradient centrifugation (Maniatis *et al.*, 1982). pBJ380 was constructed by amplifying the open reading frame of the 19-kDa US3 protein using Vent polymerase (New England Biolabs, Beverly, MA) and oligonucleotides 133 (5' CAGGAATTCTCAGCGA AGCACTGC 3') and 222 (5' AATGGATCCTTAAATAAATCGCAGACGG 3'); the resulting amplicon was inserted into the multiple cloning site of pIRESneo (Clontech).

pBJ322, 323, and 521 express the proteins encoded by the doubly spliced, singly spliced, or unspliced US3 transcripts, respectively, as in-frame fusions with enhanced green fluorescent protein. The US3-coding regions were amplified using oligonucleotide 133 in combination with oligonucleotide 143 (5' GACGGATCCACACCTGCAGT-CACCACATC 3'), 144 (5' CGTGGATCCCTTGCAGCCAG-GTAGGTTTC 3'), or 259 (5' CCAGGATCCATAATAATCG-CAGACGGGCGC 3') and p518, p517, or p516 (Tenney *et al.*, 1993), respectively, as the templates. The resulting PCR products were digested with *EcoRI* and *BamHI* and inserted into pEGFP-IRESneo 5' of the EGFP open reading frame. Junction points were sequenced to confirm the generation of an in-frame fusion of the two open reading frames.

Protein analysis

Proteins in US3-expressing cell lines were radiolabeled for 30 min with 200 μ Ci of an [^{35}S]methionine-cysteine mixture (Easy Tag Express, NEN Life Science Products, Boston, MA) using duplicate 100-mm culture dishes. One dish of cells was lysed immediately after labeling while the other dish was incubated with nonradioactive medium for 2 h prior to lysis. For immunoprecipitation, cells were placed on ice and washed two

times with cold phosphate-buffered saline. Cells were then lysed in 500 μ l of lysis buffer [50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% SDS, 100 μ g/ml PMSF, 1% Nonidet P-40, 0.5% sodium deoxycholate containing Complete protease inhibitor (Roche Biochemical, Indianapolis, IN)]. Following incubation on ice for 2 min, cell monolayers were harvested. Immunoprecipitation of the MHC class I heavy chain complexes was performed using the monoclonal antibody W6/32 (Sigma, St. Louis, MO). Polyclonal rabbit antisera generated to a NH₂-terminal region of the full-length US3 protein were generously provided by T. R. Jones (1996) and were used to immunoprecipitate US3 proteins from established cell lines.

Fluorescence microscopy

EGFP fusion proteins were detected in transfected cells using a FITC filter. The ER was labeled using a monoclonal antibody to PDI (Stressgen, Victoria, BC, Canada); the Golgi apparatus was labeled using a monoclonal antibody to human golgin-97 (Molecular Probes, Eugene, OR). Primary antibody binding was detected using Texas red conjugated goat anti-mouse antisera (Molecular Probes) as the secondary antibody. Immunofluorescence was performed as described (Colberg-Poley *et al.*, 2000). Brefeldin A was used at a concentration of 5 μ g/ml, with cells incubated for 1 h in medium containing Brefeldin A. Cells were visualized using a Nikon microscope with the appropriate fluorescence filters; images were captured using a SPOT digital camera.

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REFERENCES

- Ahn, K., Gruhler, A., Galocha, B., Jones, T. R., Wiertz, E., Ploegh, H., Peterson, P. A., Yang, Y., and Früh, K. (1997). The ER-lumenal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity* **6**, 613–621.
- Ahn, K. S., Angulo, A., Ghazal, P., Peterson, P. A., Yang, Y., and Früh, K. (1996). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA* **93**, 10990–10995.
- Biegelke, B. J. (1995). Regulation of human cytomegalovirus US3 gene transcription by a *cis*-repressive sequence. *J. Virol.* **69**, 5362–5367.
- Biegelke, B. J. (1999). Human cytomegalovirus US3 gene expression is regulated by a complex network of positive and negative regulators. *Virology* **261**, 155–164.
- Britt, W. J., and Alford, C. A. (1996). "Fields Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), 3rd ed., pp. 2493–2524. Lippincott-Raven, Philadelphia.
- Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
- Colberg-Poley, A. M., Patel, M. B., Erezo, D. P., and Slater, J. E. (2000). Human cytomegalovirus UL37 immediate-early regulatory proteins traffic through the secretory apparatus and to mitochondria. *J. Gen. Virol.* **81**, 1779–1789.
- Colberg-Poley, A. M., Santomenna, L. D., Harlow, P. P., Benfield, P. A., and Tenney, D. J. (1992). Human cytomegalovirus US3 and UL36–38 immediate-early proteins regulate gene expression. *J. Virol.* **66**, 95–105.
- Gabathuler, R., Levy, F., and Kvist, S. (1990). Requirements for the association of adenovirus type 2 E3/19K wild-type and mutant proteins with HLA antigens. *J. Virol.* **64**, 3679–3685.
- Gilbert, M. J., Riddell, S. R., Plachter, B., and Greenberg, P. D. (1996). Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* **383**, 720–722.
- Greijer, A. E., Adriaanse, H. M., Kahl, H. M., Tacken, M., Oldenburg, N. M., Sijlmans, N., van de Crommert, A., Dekkers, C. A., Sillekens, C. A., and Middeldorp, J. M. (2001). Quantitative competitive NASBA for measuring mRNA expression levels of the immediate early 1, late pp67, and immune evasion genes US3, US6 and US11 in cells infected with human cytomegalovirus. *J. Virol. Methods* **96**, 133–147.
- Hengel, H., and Koszinowski, U. (1997). Interference with antigen processing by viruses. *Curr. Opin. Immunol.* **9**, 470–476.
- Huh, G. S., Boulanger, L. M., Du, H., Fiquelme, P. A., Brotz, T. M., and Shatz, C. J. (2000). Functional requirement for class I MHC in CNS development and plasticity. *Science* **290**, 2155–2159.
- Jones, T. R., Hanson, L. K., Sum, L., Slater, J. S., Stenberg, R. M., and Campbell, A. E. (1995). Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J. Virol.* **69**, 4830–4841.
- Jones, T. R., Wiertz, E. J. H. J., Sun, L., Fish, K. N., Nelson, J. A., and Ploegh, H. L. (1996). Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. USA* **93**, 11327–11333.
- Jun, Y., Kim, E., Jin, M. R., Sung, H. C., Han, H., Geraghty, D. E., and Ahn, K. (2000). Human cytomegalovirus gene products US3 and US6 down-regulate trophoblast class I MHC molecules. *J. Immunol.* **164**, 805–811.
- Kotenko, S. V., Sacconi, S., Izotova, L. S., Mirochnitchenko, O. V., and Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc. Natl. Acad. Sci. USA* **97**, 1695–1700.
- Lee, S., Yoon, J., Park, B., Jun, Y., Jin, M., Sung, H., Kim, I.-H., Kang, S., Choi, E.-J., Anh, B., and Ahn, K. (2000). Structural and functional dissection of human cytomegalovirus US3 in binding major histocompatibility complex class I molecules. *J. Virol.* **74**, 11262–11269.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual," 1st ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sadasivan, B., Lehner, P. J., Ortmann, B., Spies, T., and Cresswell, P. (1996). Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* **5**, 103–114.
- Sciaki, N., Presley, J., Smith, C., Zaal, K. J. M., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E., and Lippincott-Schwartz, J. (1997). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* **139**, 1137–1155.
- Tenney, D. J., Santomenna, L. D., Goudie, K. B., and Colberg-Poley, A. M. (1993). The human cytomegalovirus US3 immediate-early protein lacking the putative transmembrane domain regulates gene expression. *Nucleic Acids Res.* **21**, 2931–2937.
- Tomasec, P., Braud, V. M., Rickards, C., Powell, M. B., McSharry, B. P., Gadola, S., Cerundolo, V., Borysiewicz, L. K., McMichael, A. J., and Wilkinson, G. W. (2000). Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* **287**, 1031–1033.
- Tomazin, R., Boname, J., Hegde, N. R., Lewinsohn, D. M., Altschuler, Y., Jones, T. R., Cresswell, P., Nelson, J. A., Riddell, S. R., and Johnson,

- D. C. (1999). Cytomegalovirus US2 destroys two components of the the MHC class I pathway, preventing recognition by CD4+ T cells. *Nat. Med.* **5**, 1039–1043.
- Weston, K. (1988). An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology* **162**, 406–416.
- Wiertz, E., Tortorella, D., Bogoy, M., Mothes, W., Jones, T. R., Rapaport, T. A., and Ploegh, H. (1996a). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**, 432–438.
- Wiertz, E. J. H. J., Jones, T. R., Sun, L., Bogoy, M., Geuze, H. J., and Ploegh, H. L. (1996b). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84**, 769–779.
- Ziegler, H., Muranyi, W., Burgert, H. G., Kremmer, E., and Koszinowski, U. H. (2000). The luminal part of the murine cytomegalovirus glycoprotein gp40 catalyzes the retention of MHC class I molecules. *EMBO Journal* **19**, 870–881.
- Ziegler, H., Thäle, R., Lucin, P., Muranyi, W., Flohr, T., Hengel, H., Farrell, H., Rawlinson, W., and Koszinowski, U. H. (1997). A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/*cis*-Golgi compartments. *Immunity* **6**, 57–66.